

Sildenafil reverses cardiac dysfunction in the *mdx* mouse model of Duchenne muscular dystrophy

Candace M. Adamo^a, Dao-Fu Dai^{b,1}, Justin M. Percival^{c,1}, Elina Minami^d, Monte S. Willis^e, Enrico Patrucco^a, Stanley C. Froehner^{c,2}, and Joseph A. Beavo^{a,2}

Departments of ^aPharmacology, ^bPathology, ^cPhysiology and Biophysics, and ^dMedicine, University of Washington, Seattle, WA 98195; and ^eDepartment of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC 27599

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Duchenne muscular dystrophy (DMD) is a progressive and fatal genetic disorder of muscle degeneration. Patients with DMD lack expression of the protein dystrophin as a result of mutations in the X-linked dystrophin gene. The loss of dystrophin leads to severe skeletal muscle pathologies as well as cardiomyopathy, which manifests as congestive heart failure and arrhythmias. Like humans, dystrophin-deficient mice (*mdx* mice) show cardiac dysfunction as evidenced by a decrease in diastolic function followed by systolic dysfunction later in life. We have investigated whether sildenafil citrate (Viagra), a phosphodiesterase 5 (PDE5) inhibitor, can be used to ameliorate the age-related cardiac dysfunction present in the *mdx* mice. By using echocardiography, we show that chronic sildenafil treatment reduces functional deficits in the cardiac performance of aged *mdx* mice, with no effect on normal cardiac function in WT controls. More importantly, when sildenafil treatment was started after cardiomyopathy had developed, the established symptoms were rapidly reversed within a few days. It is recognized that PDE5 inhibitors can have cardioprotective effects in other models of cardiac damage, but the present study reports a prevention and reversal of pathological cardiac dysfunction as measured by functional analysis in a mouse model of DMD. Overall, the data suggest that PDE5 inhibitors may be a useful treatment for the cardiomyopathy affecting patients with DMD at early and late stages of the disease.

cGMP | phosphodiesterase | Viagra | echocardiography

Duchenne muscular dystrophy (DMD) is a degenerative, muscle-wasting disease caused by mutations in the dystrophin gene. The total loss of dystrophin profoundly impacts skeletal muscle and causes impaired respiratory function, primarily in older boys (1, 2). With improved noninvasive respiratory support, patients with DMD experience increased lifespan and quality of life, but also a higher incidence of complications and eventual mortality from cardiomyopathy (3). Cardiomyopathy is a delayed symptom of the disease that usually develops by the second decade of life, with more than 90% of patients presenting clinical symptoms by 18 y of age (1). Loss of cardiac dystrophin eventually leads to dilated cardiomyopathy, which manifests as congestive heart failure in at least 20% of patients (1). Similarly, approximately 90% of patients with Becker muscular dystrophy, a milder form of the disease, die of cardiac failure (1). Currently, angiotensin converting enzyme inhibitors and β -blockers are used to treat heart failure associated with DMD. Although these medications show some benefit for patients with systolic heart failure, they have been unsuccessful in treating dystrophic patients with features of systolic and diastolic dysfunction (4). These findings highlight the need for treatments that slow the development of cardiomyopathy in DMD and improve cardiac function in older patients with established cardiomyopathy.

NO-cGMP signaling pathways may provide a new potential target for therapeutic intervention in DMD. The NO-cGMP pathways are critical regulators of cardiac, smooth, and skeletal muscle contractile function. NO is generated by Ca^{2+} -sensitive NOS and increases the production of cGMP by stimulating soluble guanylyl cyclase activity. Loss of dystrophin prevents normal nNOS expres-

sion and/or signaling in all (skeletal, smooth and cardiac) muscle systems (2). In fact, aberrant neuronal NOS (nNOS) signaling is a common characteristic of many neuromuscular diseases (5). Previous studies have shown that stimulation of cGMP synthesis by overexpression of cardiac-specific nNOS reduces impulse-conduction defects in dystrophin-deficient (*mdx*) mice (6, 7). Similarly, increased particulate guanylyl cyclase activity in young *mdx* mice has also been shown to decrease susceptibility to cardiac damage during sympathetic stress (8). These data suggest that reduced NO-cGMP signaling is a key contributor to DMD cardiac pathogenesis. Therefore, it seemed possible that restoration of NO signaling, or the downstream consequences of nNOS signaling—particularly increased cGMP second messenger levels—may provide therapeutic benefit to dystrophic hearts. In this study, we tested whether blockade of cGMP breakdown using a phosphodiesterase 5 (PDE5) inhibitor might produce similar cardioprotective effects.

Sildenafil citrate is a cGMP-specific PDE5 inhibitor widely used to treat erectile dysfunction (as Viagra; Pfizer) and pulmonary hypertension (as Revatio; Pfizer). It has been previously suggested that PDE5 inhibitors may have cardioprotective effects in the *mdx* mouse (8). These investigators reported that acute injections of sildenafil reduced Evans blue dye uptake during mechanical stress (8). However, it was not clear how this result in very young *mdx* mice related to older *mdx* mice with established cardiac dysfunction. We report here both long-term protection and a rapid reversal of established pathological cardiac dysfunction in the *mdx* mouse model of DMD following chronic sildenafil treatment, some of which have been presented in abstract form (9). Chronic sildenafil treatment prevents functional deficits in the left ventricular performance of aged *mdx* mice. Furthermore, when sildenafil treatment is initiated after the cardiomyopathy has developed, the established symptoms are rapidly reversed. Overall, the data suggest that PDE5 inhibitors may be an effective treatment for DMD-associated cardiomyopathy at early and late stages of the disease.

Results

Protection of Global Cardiac Function by Long-Term Sildenafil Treatment. We used conventional echocardiography and tissue Doppler analysis to monitor the development of left ventricular dysfunction in aging *mdx* mice. Both the myocardial performance index (MPI) and ratios of early diastolic velocity (Ea) to peak velocity with atrial contraction (Aa) were calculated. MPI is a sensitive measure of left ventricular systolic and diastolic performance, whereas the Ea/Aa largely reflects diastolic function. The majority of patients with

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¹D.-F.D. and J.M.P. contributed equally to this work.

²To whom correspondence may be addressed. E-mail: froehner@u.washington.edu or beavo@u.washington.edu.

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DMD exhibit diastolic dysfunction and impaired myocardial performance, which can be identified by increased MPI (10). This dysfunction usually precedes the onset of systolic heart failure and dilated cardiomyopathy (11). *Mdx* mice show these same echocardiographic abnormalities (12, 13).

of long-term cardiac remodeling. Instead, the mechanism underlying improved cardiac performance occurs relatively rapidly in just a matter of days. This is not consistent with the beneficial effects of sildenafil being directly caused by long-term changes in fibrosis or rapid acute changes in cardiovascular tone.

Diastolic Dysfunction Is Improved After Sildenafil Treatment in *mdx* Mice. As the improvement by sildenafil in the MPI could be a result of effects on systolic or diastolic function, we measured the Ea/Aa using tissue Doppler imaging to more directly evaluate diastolic function in *mdx* mice. This parameter largely reflects the diastolic (chamber relaxation and filling) capacity of the left ventricle. As shown in Fig. 2, diastolic dysfunction (indicated by Ea/Aa <1) was observed in *mdx* mice as early as 8 mo of age (Fig. 2B). Moreover, chronic sildenafil treatment reduced the progression of diastolic dysfunction in *mdx* mice through 15 mo of age. Similarly, sildenafil initiated at 12 mo of age, after the dysfunction had developed, also largely reversed the diastolic dysfunction by 15 mo (Fig. 2B). This result is consistent with the effect of sildenafil on improvement seen in the MPI and therefore suggests that diastolic dysfunction is a major component of the impaired MPI observed in 11- to 13-mo-old *mdx* mice.

Sildenafil Alters Cardiac Dimensions. Cardiac remodeling after injury can result in hypertrophy, increased fibrosis and systolic dysfunction of the heart. Cardiomyopathy in *mdx* mice is characterized by slow, progressive cell death, followed by compensatory hypertrophy of remaining cardiomyocytes and eventual replacement fibrosis. We used M-mode echocardiography to determine left ventricular dimensions in conscious *mdx* mice. Although the *mdx*^{ScSn} strain develops overt cardiac hypertrophy (12), this is much less apparent in the *mdx4cv* strain used in all these studies (Table 1). Nevertheless, by 12 mo of age, the left ventricular walls of *mdx4cv* mice were thicker and the left ventricular mass index (LVMI) larger compared with sildenafil-treated *mdx4cv* mice (Table 1). This effect of sildenafil on wall thickness, in addition to the acute effects on diastolic function, suggests that sildenafil may also have protective effects on some aspects of cardiac remodeling. However, we found no differences in the percent of fractional shortening (FS) percentage of 12-mo-old, conscious *mdx* mice compared with WT controls or sildenafil-treated *mdx* mice. There was also no effect on heart rate (Table 1). This indicates a lack of major systolic dysfunction in these animals up to 12 mo of age. Although systolic dysfunction may develop later in life, it appears that diastolic dysfunction plays a more prominent role in the cardiomyopathy seen in *mdx4cv* mice.

GSK-3 β Phosphorylation Is Up-Regulated After Long-Term Sildenafil Treatment. The unexpected rapid reversal of the cardiac dysfunction caused by sildenafil greatly constrained our ideas about the molecular mechanism(s) by which the drug might be acting. It is likely that

several molecular mechanism(s) could be altered by sildenafil that may lead to improved cardiac function. In studies of the protective effects of this drug on cardiac ischemia/reperfusion injury, sildenafil has been reported to up-regulate cGMP-dependent protein kinase G (PKG) and PKG-dependent phosphorylation of glycogen synthase kinase 3 β (GSK-3 β) (14). As this effect of sildenafil was potentially relevant to the time course observed for the reversal studies, we measured PKG protein expression and GSK-3 β phosphorylation in the ventricular tissue from 16-mo-old *mdx* mice after long-term sildenafil treatment. As shown in Fig. 3, both increased PKG expression and GSK-3 β phosphorylation were seen (Fig. 3A–C). Thus, long-term sildenafil treatment of dystrophin-deficient animals may initiate protective mechanisms similar to those responsible for protection from ischemia-reperfusion injury. However, additional studies will be needed to prove this hypothesis.

Discussion

We have investigated the impact of long-term oral administration of sildenafil on prevention of cardiac dysfunction and restoration of MPI in *mdx* mice. This study identifies an apparent strong therapeutic effect of sildenafil on the abnormal MPI and Ea/Aa ratios in *mdx* mice. To our knowledge, this is the first report of rapid reversal by a drug treatment of the functional symptoms seen in the established cardiac dysfunction that occurs in *mdx* mice. Although several studies have described protective effects of sildenafil against other forms of cardiac myopathy and hypertrophy (2), to our knowledge, the only study to date on the effects of sildenafil on dystrophic cardiac dysfunction has reported that repeated injections of sildenafil in very young *mdx* mice can reduce cardiomyocyte susceptibility to mechanical injury (8). The same study also reported a reduction in several possible early markers of cardiomyopathy seen before the onset of cardiac dysfunction (8). It is well established that older *mdx* mice show an increase in cardiac mass and diastolic dysfunction (12, 15–17), providing us an excellent model to examine the effects of sildenafil on established cardiomyopathy. In fact, one study has reported the development of cardiac hypertrophy in *mdx* mice as early as 16 to 21 wks (12), suggesting that the reduced wall thickness we see in the chronically sildenafil-treated 12-mo-old *mdx* mice may be indicative of a long-term, sustained improvement.

Interpretation of the possible mechanism(s) by which sildenafil might be improving the MPI and Ea/Aa ratio are made difficult by the fact that little is known about why these echocardiographic parameters are altered in the *mdx* mouse heart. In principle, the cardioprotective effects of sildenafil could be caused by enhanced systolic or diastolic function. We observed no systolic dysfunction in 12-mo-old *mdx* mice, which is consistent with findings from Jearawiriyapaisarn et al. (12). Previous reports suggest that systolic dysfunction may develop with age (15–18). The reasons for these discrepancies are not known, but may be related to differences in the *mdx* strains used or perhaps other differences in genetic backgrounds. Because we do not see systolic dysfunction in *mdx* mice, the poor cardiac performance measured by MPI seems most likely to be a result of diastolic dysfunction. This interpretation is supported by tissue Doppler imaging measurements of diastolic function. A diastolic Ea/Aa ratio is typically at least 1, with values less than 1 reflecting abnormal wall relaxation/filling. The changes we see in Ea/Aa ratio upon sildenafil treatment reinforce the idea that the major cardiac dysfunction in 12- to 15-mo-old *mdx* mice is diastolic in nature. As wt controls do not display cardiac dysfunction, we do not see an effect with sildenafil treatment. Currently, there are no validated therapeutic approaches that effectively target diastolic dysfunction (19). Thus, cGMP enhancement by sildenafil may be an effective treatment of other forms of diastolic dysfunction.

Neither the target cells nor molecular mechanism(s) for sildenafil's cardioprotective effects are clear. Previous reports suggest that cGMP-mediated activation of PKG signaling can occur in cardiomyocytes (20). Increased PDE5 expression also has been

Table 1. Cardiac dimensions and systolic function of treated 12-mo-old *mdx* mice

Parameter	WT	<i>mdx</i>	<i>mdx</i> (sildenafil)
No. of mice	7	18	12
BW, g	35.59 \pm 0.6	34.12 \pm 0.2	34.47 \pm 0.2
IVSd, mm	1.05 \pm 0.01	1.06 \pm 0.01	0.92 \pm 0.01*
LVIDd, mm	3.22 \pm 0.04	3.16 \pm 0.02	3.32 \pm 0.02
PWd, mm	0.95 \pm 0.02	1.03 \pm 0.01	0.89 \pm 0.01*
FS, %	52.74 \pm 0.5	47.23 \pm 0.3	51.78 \pm 0.5
LVMI, mm/g	3.24 \pm 0.1	3.48 \pm 0.03	3.00 \pm 0.03*
HR, beats/min	629 \pm 7.0	616.28 \pm 3.6	597.43 \pm 8.9

BW, body weight; HR, heart rate. Values are means \pm SE analyzed by two-way ANOVA.

*P < 0.05 *mdx* compared with *mdx* (sildenafil).

reported in rat cardiomyocytes during cardiac hypertrophy and remodeling, suggesting that sildenafil may act directly on cardiomyocytes (21). However, others have not observed this effect in cardiomyocytes isolated from mice (22). Even though it is unclear which cell type is targeted by sildenafil, total heart extracts from patients with end-stage congestive heart failure have increased PDE5 expression via a mechanism involving oxidative stress (23), and a recent study has shown an increase in oxidative stress in the hearts of young *mdx* mice (24). Whether this increase in PDE5 expression takes place in cardiomyocytes, myofibroblasts, or vascular smooth muscle cells is still to be determined.

In addition, one cannot rule out other PDEs as a potential target for sildenafil. We detected a mean free plasma concentration of sildenafil to be approximately 70 nM, sufficient to greatly inhibit PDE5 (Fig. S1), but given the high rate of metabolism of sildenafil in mice, peak levels of the drug may be high enough to inhibit other PDEs such as PDE1C. With an IC_{50} of 280 nM, partial inhibition of PDE1C could explain some of the cardioprotective effects seen in this study, especially as PDE1C is highly expressed in cardiomyocytes and vascular smooth muscle. Future experiments using other PDE5 inhibitors, such as tadalafil (Cialis; Eli Lilly) which does not inhibit PDE1C, could provide insight on the molecular mechanism(s) by which sildenafil has a cardioprotective effect in *mdx* mice.

Finally, at least some of the beneficial effects of long-term treatment by sildenafil observed in this study could be explained by PKG-dependent phosphorylation of GSK-3 β (Fig. 3) (14, 25, 26). Phosphorylation of GSK-3 β (Ser9) has been associated with cardioprotection in ischemia/reperfusion models of cardiac damage, in which treatment with sildenafil decreased infarct size in an AKT-independent manner (14). Other studies have shown that GSK-3 β kinase activity is inhibited by PKG-dependent phosphorylation, resulting in decreased apoptosis and fibrosis and ultimately increased contractility (27). The improvements in cardiac function seen with sildenafil in the present studies may result, at least in part, from similar mechanisms. However, given the short time scale of the reversal, additional mechanisms are also likely to be important, such as effects on mitochondrial biogenesis and function (28).

All the possible mechanisms discussed here assume that the cardioprotective effects result from actions on the cardiomyocytes. However, cardiomyocyte-independent mechanisms also could be responsible for part or all the improved cardiac performance. As PDE5 is highly expressed in vascular smooth muscle, sildenafil-mediated vasodilation may reduce cardiac afterload and improve cardiac performance. Mice lacking nNOS or dystrophin cannot properly regulate blood flow during exercise and have impaired vascular perfusion as well as functional ischemia (6). Thus, sildenafil-mediated enhancement of blood flow in exercised dystrophic muscle (5) may also contribute to the therapeutic effect of the drug on cardiac function. This idea is consistent with the hypothesis that contracting muscles, such as the heart, require enhanced NO-cGMP signaling, such as might be provided by PDE5 inhibition.

Regardless of its molecular mechanisms of action, sildenafil citrate is currently approved for short-term human use in treatment of erectile dysfunction (Viagra) and long-term use in treatment of pulmonary hypertension (Revatio). Our study used a comparable dose of sildenafil as those used for chronic treatment of pulmonary hypertension (Fig. S1). To our knowledge, this study is the first to report a treatment using an Food and Drug Administration-approved drug that can reverse established left ventricular dysfunction within 3 d of drug administration and maintain normal function (based on pulse-wave Doppler analysis of MPI) for a minimum of 3 mo. Although PDE5 inhibitors will certainly not cure DMD, the current studies suggest that they could be used in combination with current or future therapies. In fact, targeting skeletal muscle repair without cardiac treatment exacerbates damage to the heart, emphasizing the importance for a cardiac-specific treatment (29). Other developing therapies for DMD in-

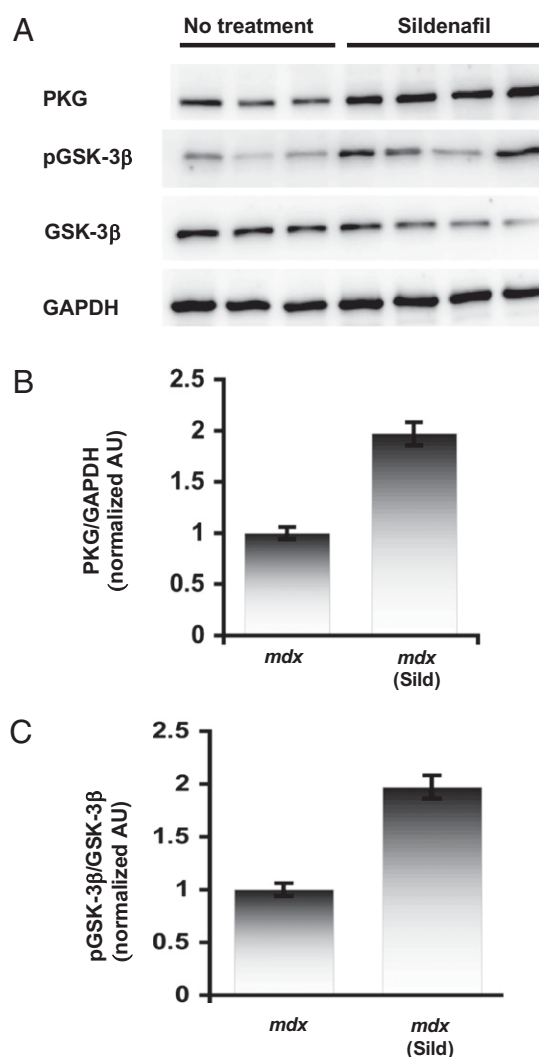


Fig. 3. Sildenafil-mediated cardioprotection in 16-mo-old *mdx* hearts is associated with inactivation of GSK-3 β . (A) Representative Western blots showing relative abundance of PKG, pGSK-3 β , GSK-3 β , and GAPDH. (B) PKG protein expression normalized to GAPDH and (C) pGSK-3 β protein expression normalized to total GSK-3 β ; $n = 7$ mice/group; * $P < 0.05$ analyzed by unpaired t test.

clude modulation of exon splicing (i.e., exon skipping) and gene transfer (13). Although these technologies could be a potential cure for DMD, their mainstream use is likely to be years away. In summary, the cardioprotective effects we see suggest that sildenafil can be useful as a preventive therapy for the cardiomyopathy that develops in muscular dystrophy and may also effectively reverse established diastolic dysfunction seen in patients with DMD.

Methods and Materials

Animal Models. All animals used in these studies were of the *mdx4cv* strain. *mdx4cv* breeding pairs were a gift from Jeff Chamberlain (University of Washington, Seattle). The breeding pairs consisted of *mdx* mice (B6Ros.Cg-Dmd^{mdx4cv}/J) and WT mice (C57BL/6). All animals were housed in a specific pathogen-free facility and the study was approved by the University of Washington Institutional Animal Care and Use Committee. Male mice were weaned at 4 wks of age, some of which were immediately started on chronic sildenafil treatment.

Oral Administration of PDE5A Inhibitor. Sildenafil citrate (100-mg tablets; Viagra; Pfizer) was dissolved in acidified water (pH 3.0) to a final concentration of 400 mg/L, sterile filtered, and given ad libitum, resulting in the

ingestion of approximately 80 mg/kg/d. The IC_{50} for sildenafil inhibition of 50% of PDE5A is 10 nM.

Echocardiography. Transthoracic, 2D-guided M-mode echocardiography was obtained at the level of the papillary muscles on conscious mice at 12 mo of age. Every attempt was made to keep the operator blinded to mouse type and treatment, but there were noticeable phenotypic differences. Thus, analysis was performed blinded and on separate days from acquisition. For conscious echocardiography (Vevo 770; VisualSonics), a 30-MHz high-frequency transducer was used (RMV 707B). The mouse was held in the prone position by restraining their limbs with elastic bands. Mice were trained 1 d before collecting experimental images by applying gel and touching the chest with the probe for at least 5 min. Cardiac dimensions, heart rate, and the percentage of fractional shortening (FS) were obtained. FS was calculated as $(LVIDd - LVIDs) / LVIDd \times 100\%$, where LVIDd is the left ventricular internal dimension at end-diastole and LVIDs is the left ventricular internal dimension at end-systole. LVMI was calculated by $1.04 \times (IVSd + LVIDd + PWD)^3 - (LVIDd)^3$, where IVSd is the interventricular septal thickness at end-diastole, PWD is the posterior wall thickness at end-diastole, and LVIDs is the left ventricular internal dimension at end-systole.

Anesthetized Doppler analyses were performed by using an Acuson CV-70 unit (Siemens) equipped with a 13-MHz probe. Isoflurane 0.5% to 1% mixed with O_2 was administered to provide adequate sedation while minimizing cardiac suppression during echocardiography. An increase in MPI is an indication that a greater fraction of systole is spent to cope with the pressure changes during isovolumic phases, which has been shown to be a sign of left ventricular systolic and diastolic dysfunction (30). The MPI was obtained by using pulse-wave Doppler imaging of mitral valve inflow from the apical four-chamber view as $(IVCT + IVRT) / LVET$, where IVCT is the isovolumic contraction time, IVRT is the isovolumic relaxation time, and LVET is left ventricular ejection time. For the 2-wk sildenafil treatment regimen (Fig. 2C), the MPI was measured using the VisualSonics Vevo 770 unit with a 30-MHz high-frequency transducer (RMV 707B) to obtain higher resolution for analysis. For tissue

Doppler measurements, sample volume was placed within the left posterior wall of the myocardium at the level of the papillary muscles to align the sample volume parallel with the Doppler flow by angle correction to optimally assess flow velocities. Doppler tissue peak Ea and peak Aa were used to determine the Ea/Aa ratio.

Protein Extraction and Western Blot Analysis. Frozen ventricles were ground to a powder using a mortar and pestle precooled with liquid nitrogen, and then rotor-homogenized (Ultra Turrax) in extraction buffer [100 mM Tris-HCl, pH 8, 1% SDS, 1% deoxycholate-sodium, 20 mM EDTA, plus one Protease Inhibitor Mixture Tablet (Roche) per 10 mL and one Phostop Tablet (Roche) per 10 mL protein (20 μ g)], separated by SDS/PAGE, and transferred to a PVDF membrane. The membrane was incubated overnight with primary antibodies for pGSK-3 β (Ser9), GSK-3 β , GAPDH (Cell Signaling Technology), or PKG (Calbiochem). The membrane was washed in 0.5% Tween-20 in tris-buffered saline solution, incubated with HRP-conjugated donkey anti-rabbit secondary antibody for 1 h at room temperature, then washed again. The blots were developed using the Supersignal West Femto chemiluminescence detection system (Pierce). Bands were imaged using a CCD (Alpha Innotech) and band intensity was measured using ImageJ software. GAPDH was used as sample loading control.

Statistical Analysis. Data are presented as mean \pm SE unless otherwise specified. Two-way analysis of variance followed by a post-hoc test was used to assess statistical significance.

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